(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 723 590 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
15.12.2004 Bulletin 2004/51

(21) Application number: **94931345.6**

(22) Date of filing: **13.10.1994**

(51) Int Cl.[7]: **C12N 15/57**, C12N 9/54, C12N 1/21

(86) International application number:
PCT/US1994/011562

(87) International publication number:
WO 1995/010615 (20.04.1995 Gazette 1995/17)

(54) **SUBTILISIN VARIANTS**
SUBTILISIN VARIANTEN
VARIANTS DE SUBTILISINE

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **14.10.1993 US 137240**

(43) Date of publication of application:
31.07.1996 Bulletin 1996/31

(60) Divisional application:
04024046.7

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Printed by Jouve, 75001 PARIS (FR)

Description

Field of the Invention

[0001] The present invention relates to novel carbonyl hydrolase variants having an amino acid sequence wherein a plurality of amino acid residues of a precursor carbonyl hydrolase, specifically those at positions corresponding or equivalent to residue +76 in combination with one or more of the residues selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 in *Bacillus amyloliquefaciens* subtilisin, have been substituted with a different amino acid. Such mutant/variant carbonyl hydrolases, in general, are obtained by *in vitro* modification of a precursor DNA sequence encoding a naturally-occurring or recombinant carbonyl hydrolase to encode the substitution of a plurality of these amino acid residues in a precursor amino acid sequence alone or in combination with other substitution, insertion or deletion in the precursor amino acid sequence.

Background of the Invention

[0002] Serine proteases are a subgroup of carbonyl hydrolase. They comprise a diverse class of enzymes having a wide range of specificities and biological functions. Stroud, R. Sci. Amer., **131**:74-88. Despite their functional diversity, the catalytic machinery of serine proteases has been approached by at least two genetically distinct families of enzymes: the subtilisins and the mammalian chymotrypsin related and homologous bacterial serine proteases (e.g., trypsin and *S. gresius* trypsin). These two families of serine proteases show remarkably similar mechanisms of catalysis. Kraut, J. (1977), Ann. Rev. Biochem., **46**:331-358. Furthermore, although the primary structure is unrelated, the tertiary structure of these two enzyme families bring together a conserved catalytic triad of amino acids consisting of serine, histidine and aspartate.

[0003] Subtilisin is a serine endoprotease (MW 27,500) which is secreted in large amounts from a wide variety of Bacillus species and other microorganisms. The protein sequence of subtilisin has been determined from at least four different species of *Bacillus*. Markland, F.S., et al. (1983), Honne-Sevler's Z. Physiol. Chem., **364**:1537-1540. The three-dimensional crystallographic structure of *Bacillus amyloliquefaciens* subtilisin to 2.5Å resolution has also been reported. Wright, C.S., et al. (1969), Nature, **221**:235-242; Drenth, J., et al. (1972), Eur. J. Biochem., **26**:177-181. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972), Biochemistry, **11**:2439-2449) or product complexes (Robertus, J.D., et al. (1976), J. Biol. Chem., **251**:1097-1103) have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisin (Philipp, M., et al. (1983), Mol. Cell. Biochem., **51**:5-32; Svendsen, B. (1976), Carlsberg Res. Comm., **41**:237-291; Markland, F.S. Id.) as well as at least one report wherein the side chain of methionine at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer, D.C., et al. (1965), J. Biol. Chem., **244**:5333-5338) and the side chain of serine at residue 221 converted to cysteine by chemical modification (Polgar, et al. (1981), Biochimica et Biophysica Acta, **667**:351-354.)

[0004] US Patent 4,760,025 (RE 34,606) discloses the modification of subtilisin amino acid residues corresponding to positions in *Bacillus amyloliquefaciens* subtilisin tyrosine -1, aspartate +32, asparagine +155, tyrosine +104, methionine +222, glycine +166, histidine +64, glycine +169, phenylalanine +189, serine +33, serine +221, tyrosine +217, glutamate +156 and alanine +152. US Patent 5,182,204 discloses the modification of the amino acid +224 residue in *Bacillus amyloliquefaciens* subtilisin and equivalent positions in other subtilisins which may be modified by way of substitution, insertion or deletion and which may be combined with modifications to the residues identified in US Patent 4,760,025 (RE 34,606) to form useful subtilisin mutants or variants. US Patent 5,155,033 discloses similar mutant subtilisins having a modification at an equivalent position to +225 of *B. amyloliquefaciens* subtilisin. US Patents 5,185,258 and 5,204,015 disclose mutant subtilisins having a modification at positions +123 and/or +274.

[0005] US Patent Application SN 07/898,382 discloses the modification of many amino acid residues within subtilisin, including specifically +99, +101, +103, +107, +126, +128, +135, +197 and +204. All of these patents/applications are commonly owned. US Patent 4,914,031 discloses certain subtilisin analogs, including a subtilisin modified at position +76. The particular residues identified herein and/or the specific combinations claimed herein, however, are not identified in these references.

[0006] The present invention provides subtilisin variants as set out in claim 1. Preferred subtilisin variants are as set out in claim 2.

[0007] The invention further provides DNA sequences encoding such variants, as well as expression vectors containing such variant DNA sequences.

[0008] The invention further provides host cells transformed with such vectors, as well as host cells which are capable

of expressing such DNA to produce variants either intracellularly or extracellularly.

[0009] The references discussed above are provided solely for their disclosure prior to the filing date of the instant case, and nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of priority based on earlier filed applications.

Summary of the Invention

[0010] The invention includes non-naturally-occurring subtilisin, variants having, improved wash performance characteristics as compared to the precursor subtilisin from which the amino acid sequence of the variant is derived. The precursor subtilisin may be a naturally-occurring or recombinant subtilisin. Specifically, such variants have an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor subtilisin with different amino acids.

[0011] The invention also includes variant DNA sequences encoding such subtilisin variants. These variant DNA sequences are derived from a precursor DNA sequence which encodes a Although the amino acid residues identified for modification herein are identified according to the numbering applicable to *B. amyloliquefaciens* (which has become the conventional method for identifying residue positions in all subtilisins), the preferred precursor DNA sequence useful in the present invention is the DNA sequence of *Bacillus lentus* as shown in Fig. 6 (Seq ID No.11).

Brief Description of the Drawings

[0012]

Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No.6).

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (Seq. ID No. 7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No.8). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (Seq. ID No.9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276) (Seq. ID No.10). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 depicts the construction of plasmid GGA274.

Fig. 5 depicts the construction of GGT274 which is an intermediate to certain expression plasmids used in this application.

Figs. 6A and 6B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentus* (Seq. ID No.11). The mature subtilisin protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Figs. 7A and 7B depict the DNA and amino acid sequence of a preferred embodiment of the invention (N76D/S103A/V104I) (Seq. ID No.12). The DNA in this figure has been modified by the methods described to encode aspartate at position 76, alanine at position 103 and isoleucine at position 104. The mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Fig. 8 depicts the construction of vector pBCDAICAT.

Fig. 9 depicts the construction of vector pUCCATFNA.

Fig. 10 shows the stability of a preferred mutant enzyme compared to wild-type, in a liquid detergent formulation.

[0013] Carbonyl hydrolases are enzymes which hydrolyze compounds containing

bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally-occurring carbonyl hydrolases principally include hydrolases, e.g., peptide hydrolases such as subtilisins or metalloproteases. Peptide hydrolases include α-aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

[0014] "Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally-occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein, and in US Patent 4,760,025 (RE 34,606), US Patent 5,204,015 and US Patent 5,185,258.

[0015] Subtilisins are bacterial or fungal carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein.

[0016] "Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring subtilisin amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent 4,760,025 (RE 34,606), US Patent 5,204,015 and US Patent 5,185,258.

[0017] "Non-human carbonyl hydrolases" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *Saccharomyces cerevisiae,* fungi such as *Aspergillus* sp. and non-human mammalian sources such as, for example, *bovine* sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with procaryotic and eucaryotic sources.

[0018] A "carbonyl hydrolase variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase." The precursor carbonyl hydrolases (such as a subtilisin) include naturally-occurring carbonyl hydrolases (subtilisin) and recombinant carbonyl hydrolases (subtilisin). The amino acid sequence of the carbonyl hydrolase variant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase (subtilisin) rather than manipulation of the precursor carbonyl hydrolase (subtilisin) enzyme *per se.* Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

[0019] Subtilisin variants of the invention are described in claims 1 and 2. The amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor subtilisin is *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus*.

[0020] A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

[0021] In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which sequence is known. Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

[0022] For example, in Fig. 3 the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

[0023] These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other carbonyl hydrolases such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred subtilisin precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis.*

[0024] Thus, for example, the amino acid at position +76 is asparagine (N) in both *B. amyloliquefaciens* and *B. lentus* subtilisins. In the subtilisin variant of the invention, however, the amino acid equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartate (D). A comparison of certain of the amino acid residues identified herein for substitution versus the, or the most preferred substitution for each such position is provided in Table I for illustrative purposes.

Table I

	+76	+99	+101	+103	+104	+107	+123
B. amyloliquefaciens (wild-type)	N	D	S	Q	Y	I	N
B. lentus (wild-type)	N	S	S	S	V	I	N
Most Preferred Substitution	D	D	R	A	I/Y	V	S

[0025] Equivalent residues may also be defined by determining homology at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R\ factor = \frac{\Sigma_h |Fo(h)| - |Fc(h)|}{\Sigma_h |Fo(h)|}$$

[0026] Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publi-

cation No. 0 251 446 (equivalent to US Patent Application SN 08/212,291) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

[0027] Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The carbonyl hydrolase variants of the present invention include the mature forms of carbonyl hydrolase variants, as well as the pro- and prepro-forms of such hydrolase variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase variants.

[0028] "Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a carbonyl hydrolase which when removed results in the appearance of the "mature" form of the carbonyl hydrolase. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing carbonyl hydrolase variants, specifically subtilisin variants, is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other subtilisin prosequences may be used. In Examples 1 and 2 the putative prosequence from the subtilisin from *Bacillus lentus* (ATCC 21536) was used.

[0029] A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a carbonyl hydrolase or to the N-terminal portion of a prohydrolase which may participate in the secretion of the mature or pro forms of the hydrolase. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The present invention utilizes such sequences to effect the secretion of the carbonyl hydrolase variants as defined herein. A preferred signal sequence used in the Examples comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

[0030] A "prepro" form of a carbonyl hydrolase variant consists of the mature form of the hydrolase having a prosequence operably linked to the amino terminus of the hydrolase and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

[0031] "Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

[0032] The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing subtilisin include *Bacillus subtilis* I168 (also described in US Patent 4,760,025 (RE 34,606) and US Patent 5,264,366), as well as any suitable *Bacillus* strain such as *B. licheniformis*, *B. lentus*, etc.

[0033] Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the carbonyl hydrolase variants or expressing the desired carbonyl hydrolase variant. In the case of vectors which encode the pre- or prepro-form of the carbonyl hydrolase variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

[0034] "Operably linked, " when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

[0035] The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced. The *B. lentus* gene used in the Examples was cloned as described in Example

1 of US Patent 5,185,258. The BPN' gene used in Example 5 was cloned as described in Example 1 in RE 34,606.

[0036] The cloned carbonyl hydrolase is then used to transform a host cell in order to express the hydrolase. The hydrolase gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promotor if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the hydrolase gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the hydrolase gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

[0037] The genes used in the present examples are a natural *B. lentus* gene and a natural *B. amyloliquefaciens* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase (subtilisin) may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor hydrolase (subtilisin) is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor hydrolase. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015.

[0038] Once the naturally-occurring or synthetic precursor carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in US Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of carbonyl hydrolase variants described herein.

[0039] The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase variants of the present invention, although other methods including site-directed mutagenesis may be used. First, the naturally-occurring gene encoding the hydrolase is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the hydrolase gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the hydrolase gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

[0040] Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

[0041] As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to Improved such performance, the variant enzymes of the present invention may have other modified properties such as K_m, k_{cat}, k_{cat}/K_m ratio and/or modified substrate specificity, modified proteolytic activity, and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present.

[0042] The variant carbonyl hydrolase may have altered proteolytic activity as compared to the precursor carbonyl hydrolase, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate.. Also of interest are variant enzymes having altered thermal stability and/or altered substrate specificity as compared to the precursor. The carbonyl hydrolase to be mutated is a subtilisin. These two terms are used interchangeably in this specification.

[0043] Embodiments of the invention are set forth in the Examples. These include the following specific combinations of substituted residues: N76D/V104I; N76D/S99D/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V and N76D/S101R/S103A/V104I. Also described in the Examples are all mutant combinations specifically claimed in the present invention. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus subtilisin.*

[0044] The carbonyl hydrolase variants of the invention are useful in formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the carbonyl hydrolase mutants of the invention. These include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015. The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the subtilisin variants of the present invention may be used for any purpose that native or wild-type subtilisins are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention show enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

[0045] Subtilisins of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% (preferably .1% to .5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

[0046] The addition of subtilising of the invention to conventional cleaning compositions does not create any special use limitation.

In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described subtilisin's denaturing temperature. In addition, subtilisins of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

[0047] The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

Example 1

Construction for the Expression of GG36 Gene in *B. subtilis*

[0048] The cloning and the construction for expression of the subtilisin gene from *B. lentus* was performed essentially the same as that described in US Patent 5,185,258. The plasmid GGA274 (described in Fig. 4 herein) was further modified in the following manner, as shown in Fig. 5. The PstI site that was introduced during the construction of the GGA274 plasmid was removed by the oligonucleotide directed mutagenesis described below, with an oligonucleotide having the following sequence: 5' GAAGCTGCAACTCGTTAAA 3' (Seq. ID No.1). The underlined "A" residue eliminated the recognition sequence of restriction enzyme PstI and changed the corresponding amino acid residue from alanine to threonine at position 274. Threonine at position 274 is the wild-type residue originally found in the cloned *B. lentus* subtilisin gene sequences. The DNA segment encoding subtilisin was excised from the plasmid GGA274 or its derivatives (GGT274 shown in Fig. 5) by EcoRI and BamHI digest. The DNA fragment was subcloned back into Bacteriophage M13-based vectors, such as MP19, for mutagenesis. After mutagenesis, the EcoRI and HindIII digest, followed by cloning, were performed to move the mutated subtilisin gene back into an expression plasmid like GGA274 for the expression and the recovery of mutated subtilisin proteins.

Example 2

Oligonucleotide-Directed Mutagenesis

[0049] Oligonucleotide-directed mutagenesis was performed as described in Zoller, M., et al. (1983), Methods Enzymol., **100**:468-500. As an example, a synthetic oligonucleotide of the sequence 5' GCTGCTCTAGACAATTCG 3' (Seq. ID No.2) was used to change the amino acid residue at position 76 from asparagine (N) to aspartic acid (D), or N76D. The underlined "G" and "C" residues denote changes from the wild-type gene sequence. The CA keeps the leucine at position +75 and changes the amino acid sequence to introduce an XbaI recognition site of the XbaI restriction enzyme (TCTAGA), while the change at GAC changes asparagine at +76 to aspartate.

[0050] For mutagenesis at positions 99, 101, 103 and 104, different oligonucleotides can be used depending on the

combination of mutations desired. For example, an oligonucleotide of the sequence 5' GTATTAGGGGCGGACGGTCGAGGCGCCATCAGCTCGATT 3' (Seq. ID No. 3) was used to simultaneously make the following changes: S99D; S101R; S103A and V104I in a single subtilisin molecule. Similarly, oligonucleotides of the sequence 5' TCAGGTTCGGTCTCGAGCGTTGCCCAAGGATTG 3' (Seq. ID No.4) and 5' CACGTTGCTAGCTTGAGTTTAG 3' (Seq. ID No.5) were utilized to generate I107V and N123S, respectively. Again, the underlined residues denote changes from wild-type sequences which produced desired changes either in amino acid sequences or restriction enzyme recognition sequences.

Example 3

Proteolytic Activity of Subtilisin Variants

[0051] Following the methods of Example 2, the variants listed in Table III were made. Proteolytic activity of each of these subtilisin variants is shown in Table II. The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were measured for hydrolysis of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in P. Bonneau, et al. (1991) J. Am. Chem. Soc., Vol. 113, No. 3, p. 1030. Briefly, a small aliquot of subtilisin variant stock solution was added to a 1 cm cuvette containing substrate dissolved in 0.1M Tris-HCL buffer, pH 8.6, and thermostated at 25°C. The reaction progress was followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm. Kinetic parameters were obtained by using a non-linear regression algorithm to fit the reaction .velocity and product concentration for each reaction to the Michaelis-Menten equation.

Table II

Kinetic Parameters k_{cat}, K_M and k_{cat}/K_M Measured for *Bacillus lentus* Subtilisin and Variants			
Enzyme	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M ($s^{-1}M^{-1}$)
B. lentus Subtilisin	170	0.00078	$2.18x10^5$
N76D	219	0.0008	$2.74x10^5$
N76D/S99D	88	0.00061	$1.44x10^5$
N76D/S103A	400	0.0014	$2.86x10^5$
N76D/V104I	459	0.0011	$4.17x10^5$
N76D/I107V	219	0.0011	$1.99x10^5$
N76D/N123S	115	0.0018	$6.40x10^4$
N76D/S99D/S101R	146	0.00038	$3.84x10^5$
N76D/S99D/S103A	157	0.0012	$1.31x10^5$
N76D/S99D/V104I .	247	0.00097	$2.55x10^5$
N76D/S101R/S103A	405	0.00069	$5.90x10^5$
N76D/S101R/V104I	540	0.00049	$1.10x10^6$
N76D/S103A/V104I	832	0.0016	$5.20x10^5$
N76D/V109I/I107V	497	0.00045	$1.10x10^6$
N76D/V104Y/I107V	330	0.00017	$1.90x10^6$
N76D/V104I/N123S	251	0.0026	$9.65x10^4$
N76D/I107V/N123S	147	0.0035	$4.20x10^4$
N76D/S99D/S101R/S103A	242	0.00074	$3.27x10^5$
N76D/S99D/S101R/V104I	403	0.00072	$5.60x10^5$
N76D/S99D/S103A/V104I	420	0.0016	$2.62x10^5$
N76D/S101R/S103A/V104I	731	0.00065	$1.12x10^6$
N76D/S103A/V104I/N123S	321	0.0026	$1.23x10^5$
N76D/V104I/I107V/N123S	231	0.003	$7.70x10^4$
N76D/S99D/S101R/S103A/V104I	624	0.00098	$6.37x10^5$
N76D/S99D/S103A/V104I/N123S	194	0.0043	$4.51x10^4$
N76D/S99D/S101R/S103A/V104I/N123S	311	0.0023	$1.35x10^5$

[0052] The results listed in Table II indicate that all of the subtilisin variants tested retain proteolytic activity. Further, detailed analysis of the data reveal that proteolytic activity was significantly altered for *Bacillus lentus* subtilisin by the various combinations of substitutions at amino acid residues equivalent to positions 76, 99, 101, 103, 104, 107 and

123 in *Bacillus amyloliquefaciens*.

Example 4

Thermal Stability of Subtilisin Variants

[0053] A comparison of thermal stability observed for *Bacillus lentus* subtilisin and the variants of the present invention made by the process of Example 2 is shown in Table III. Purified enzyme, 15 ug/ml in 0.1 M glycine 0.01% Tween-80 pH 10.0, with or without 50 mM $CaCl_2$, was aliquotted into small tubes and incubated at 10°C for 5 minutes, 10°C to 60°C over 1 minute, and 60°C for 20 minutes. Tubes were then placed on ice for 10 minutes. Aliquots from the tubes were assayed for enzyme activity by addition to 1 cm cuvettes containing 1.2 mM of the synthetic peptide substrate succinyl-L-ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity was followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time. Data are presented as percent activity prior to heating. The results listed in Table III indicate that a vast majority of variants exhibit thermal stability comparable to *Bacillus lentus* subtilisin (24 out of 26) in the test condition with 50mM $CaCl_2$ added. In the test condition without 50mM $CaCl_2$ added, a vast majority of variants (19 out of 26) are significantly more stable than *Bacillus lentus* subtilisin. Further, the variants N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V and N76D/S101R/S103A/V104I are significantly more stable than the single substitution variant N76D in the test condition without 50mM $CaCl_2$ added.

Table III

Thermal Stability Measured for *Bacillus lentus* Subtilisin and Variants At pH 10, 60°C, +/- 50mM $CaCl_2$ Added		
Enzyme	% Initial Activity Remaining	
	- $CaCl_2$	+ $CaCl_2$
B. lentus Subtilisin	2	96
N76D	34	97
N76D/S99D	49	98
N76D/S103A	26	92
N-76D/V104I	58	98
N76D/I107V	32	96
N76D/N123S	0	97
N76D/S99D/S101R	30	100
N76D/S99D/S103A	36	100
N76D/S99D/V104I	48	97
N76D/S101R/S103A	26	100
N76D/S101R/V104I	38	100
N76D/S103A/V104I	58	100
N76D/V104I/I107V	60	97
N76D/V104Y/I107V	48	74
N76D/V104I/N123S	0	98
N76D/I107V/N123S	16	100
N76D/S99D/S101R/S103A	38	100
N76D/S99D/S101R/V104I	33	100
N76D/S99D/S103A/V104I	38	98
N76D/S101R/S103A/V104I	40	99
N76D/S103A/V104I/N123S	1	98
N76D/V104I/I107V/N123S	3	99
N76D/S99D/S101R/S103A/V104I	36	99
N76D/S99D/S103A/V104I/N123S	2	95
N76D/S99D/S101R/S103A/V104I/N123S	0	100

Example 5

Oligonucleotide-Directed Mutagenesis with

Single-Stranded DNA Template Generated from Phagemid

A. Construction of *B. lentus* Variants

[0054] The mutagenesis protocol was essentially the same as described above in Example 2. The single-stranded DNA template was generated by phagemid method. To construct the phagemid vector for generating the single-stranded DNA template we first constructed the vector pBCDAICAT. The flow chart of vector construction is outlined in Figure 8. First, the *ClaI* to *ClaI* fragment encoding the CAT gene from pC194 plasmid (Horinouchi, S. and Weisblum, B., J. Bacteriol., **150**:8-15, 1982) was cloned into the *AccI* site of polylinker region of pUC19 (New England BioLabs, Beverly, MA) to make plasmid pUCCHL and the *EcoRI-DraI* 0.6 KB fragment from the 5' end of the GG36DAI encoding DNA was cloned into the *EcoRI* and *EcoRV* sites of pBSKS plasmid (Stratagene, Inc., San Diego, CA) to make pBC2SK5. The single *EcoRI* site of the plasmid pBC2SK5 was eliminated by *EcoRI* digestion, followed by filling in catalyzed-by-T4 DNA polymerase, and religation to generate the plasmid pBC2SK-5R which does not have the *EcoRI* site. The *EcoRI-DraI* fragment which was cloned into pBCSK-5R was isolated as a *PstI-HindIII* fragment and cloned into the *PstI-HindIII* site of the pUCCHL (part of the polylinker of pUC19) to generate plasmid pUCCHL5R. The encoding sequence of GG36DAI gene was excised as an *EcoRI-BamHI* fragment and cloned into the *EcoRI-BamHI* sites of pUCCHL5R to make pUCCAT. The large *EcoRI-HindIII* fragment of pUCCAT was then cloned into the *EcoRI* and *HindIII* sites of BS2KS+ to generate the plasmid pBCDAICAT.

[0055] To generate single-stranded DNA, *E. coli*-containing pBCDAICAT were infected with phage R408 (obtained from Stratagene, San Diego, CA) following the protocol described in Russel, M., Kidd, S. and Kelley, M.R., GENE 45: 333-338, 1986. Once the single-stranded DNA template was available, standard mutagenesis methods as described above in Example 2 were carried out. The construction of certain mutants is detailed below for illustrative purposes.

[0056] For the construction of *B. lentus* (GG36) N76D/S103A/V104I/L217H, an *EcoRI-BamHI* DNA fragment encoding GG36 N76D/S103A/V104I was used in the construction of pUCCAT (see Fig. 8) to generate the plasmid pBCDAICAT. After the single-stranded DNA template was made following the protocol described above, a mutagenesis primer with the following sequence

```
                                    * *** ** x ClaI
5' TAT GCC AGC CAC AAC GGT ACT TCG ATG GCT 3' (Seq. ID No.13)
```

was used to make the L217H. As before, the underlined residues denote the nucleotide changes that were made and the x *ClaI* indicates that the existing *ClaI* site was eliminated after the mutagenesis. The mutagenesis protocol was as described in Example 2. After the mutagenesis, plasmid DNA was first screened for the elimination of the *ClaI* site and those clones missing the *ClaI* site were subjected to DNA sequence analysis to verify the DNA sequence which made the L to H change at the 217th amino acid residue.

B. Construction of BPN' Variants and their Expression in *B. subtilis*

[0057] The construction of *B. amyloliquefaciens* (BPN') N76D/Q103A/Y104I/Y217L was done in a similar fashion except in two consecutive steps. N76D was first introduced into BPN' Y217L to make BPN' N76D/Y217L and a second mutagenesis was done to convert BPN' N76D/Y217L to BPN' N76D/Q103A/Y104I/Y217L. To generate the single-stranded DNA template for the first mutagenesis, an *EcoRI-BamHI* fragment encoding BPN' Y217L subtilisin (derived from the Y217L plasmid described in Wells, J., et al., PNAS, 84, 5167, 1087) was used to construct a plasmid pUCCATFNA (see Fig. 9). The pUCCATFNA plasmid containing BPN' Y217L was used to construct the pBCFNACAT plasmid (Fig. 9). Single-stranded DNA was generated as described above. To generate BPN' N76D/Y217L, an oligonucleotide primer with the sequence

```
                                * *** ** x XbaI
5' C ACA GTT GCG GCT CTA GAT AAC TCA ATC GGT G 3' (Seq. ID
No.14)
```

was used to generate the change N76D. Single-stranded DNA was then prepared from the pBCFNACAT plasmid

containing the BPN' N76D/Y217L (the pBCFNACAT plasmid after N76D mutagenesis) and mutagenized with another oligonucleotide with the sequence

```
                                    * *** ** x PvuII
5' GCT GAC GGT TCC GGC GCT ATT AGT TGG ATC ATT 3' (Seq. ID No.15)
```

to obtain BPN' N76D/Q103A/Y109I/Y217L. All steps involved in the cloning, the single-stranded DNA preparation, the mutagenesis, and the screening for mutants were carried out as described above.

[0058] Expression of the BPN' gene and its variants were achieved by integrating plasmid DNA (pBCFNACAT containing the different variants' BPN' gene) directly into a protease-deficient strain of *Bacillus subtilis* as described in RE 34,606.

[0059] Numerous variants were made as per the teachings of Examples 2 and 5. Kinetics data and stability data were generated for such variants. The kinetics data were generated using the methods described in Example 3 and are provided in Table IV. The stability data were generated as detailed herein. Results are shown in Table V.

Thermal Stability Assay Procedure

[0060] Purified enzyme was buffer-exchanged into 0.1 M glycine pH 10.0, 0.01% Tween-80 by applying the enzyme to a column consisting of Sephadex G-25 equilibrated with this buffer and eluting the enzyme from the column using the same buffer.

[0061] To a tube containing 0.1 M glycine, 0.01% Tween-80 pH 10.0 thermostatted at 60°C, the buffer-exchanged enzyme was added to give a final enzyme concentration of 15 ug/ml.

[0062] Aliquots were removed from the 60°C incubation at various times and immediately assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity was followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time.

[0063] Half-life, which is the length of time required for 50% enzyme inactivation, was determined from the first-order plot of reaction velocity as a function of the time of incubation at 60°C.

[0064] The data are presented in Table V as percent of the half-life determined for *Bacillus lentus* subtilisin (GG36) under identical conditions.

Table IV

Enzyme	**kcat** (s^{-1})	**KM** (mM)	**kcat/KM** ($s^{-1}M^{-1}$)
B. lentus subtilisin	170	0.78	2.20E+05
N76D/S103G/V104I*	380	1.4	2.70E+05
N76D/S103A/V104F	730	0.33	2.20E+06
N76D/S103A/V104N	790	2.8	2.80E+05
N76D/S103A/V104S	170	0.83	2.00E+05
N76D/S103A/V104T	370	1.9	2.00E+05
N76D/S103A/V104W	880	0.31	2.80E+06
N76D/S103A/V104Y	690	0.5	1.40E+06
K27R/N76D/V104Y/N123S	500	1.2	4.20E+05
N76D/S101G/S103A/V104I*	620	1.3	4.80E+05
N76D/S103A/V104I/S105A*	550	1.3	4.20E+05
N76D/S103A/V104I/S105D*	440	1.7	2.60E+05
N76D/S103A/V104T/I107A*	120	5.7	2.10E+04
N76D/S103A/V104T/I107L*	310	3.2	9.70E+04
N76D/S103A/V104I/L126A	90	2.2	4.10E+04
N76D/S103A/V104I/L126F	180	1.9	9.50E+04
N76D/S103A/V104I/L126I	100	2.4	4.20E+04
N76D/S103A/V109I/L126V	64	3.2	2.00E+04

Table IV (continued)

Enzyme	kcat (s^{-1})	KM (mM)	kcat/KM ($s^{-1}M^{-1}$)
N76D/S103A/V104I/S128G*	560	1.7	3.30E+05
N76D/S203A/V104I/S128L*	430	3.8	1.10E+05
N76D/S103A/V104I/L135A	140	0.76	1.80E+05
N76D/S103A/V104I/L135F	390	0.69	5.70E+05
N76D/S103A/V104I/L135I	110	0.73	1.50E+05
N76D/S103A/V104I/L135V	140	0.86	1.60E+05
N76D/S103A/V104I/S156E*	170	2.6	6.50E+04
N76D/S103A/V104I/S166D*	160	3.5	4.60E+04
N76D/S103A/V104I/D197E	510	1.4	3.60E+05
N76D/S103A/V104I/N204A*	530	1.1	4.80E+05
N76D/S103A/V104I/N204G*	580	1.4	4.10E+05
N76D/S103A/V104I/N204C*	370	1.3	2.90E+05
N76/S103A/V104I/P210I*	500	1.2	4.20E+05
N76D/ST03A/V104I/L217H*	80	0.63	1.30E+05
N76D/S103A/V104I/M222A	70	3.1	2.30E+04
N76D/S103A/V104I/M222S	80	3.1	2.60E+04
N76D/S103A/V104I/T260P	660	1.5	4.40E+05
N76D/S103A/V104I/S265N	590	1.3	4.50E+05
K27R/N76D/V104Y/I107V/N123S	220	1.4	1.60E+05
K27R/N76D/V104Y/N123S/D197E	430	1.1	3.90E+05
K27R/N76D/V104Y/N123S/N204C	400	1.1	3.60E+05
K27R/N76D/V104Y/N123S/Q206L	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/S216V	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/N218S	760	0.98	7.80E+05
K27R/N76D/V104Y/N123S/T260P	410	1.2	3.40E+05
K27R/N76D/V104Y/N123S/T274A	390	1	3.90E+05
N76D/S103A/V104I/L126F/S265N	170	2.1	8.10E+04
N76D/S103A/V104I/S156E/S166D*	40	6.3	6.40E+03
K27R/N76D/V104Y/N123S/G195E/G197E	410	0.98	4.20E+05
K27R/N76D/V104Y/N123S/G195E/N218S	540	0.66	8.20E+05
K27R/N76D/V104Y/N123S/D197E/N218S	770	0.79	9.80E+05
K27R/N76D/V104Y/N123S/N204C/N218S	610	0.99	6.20E+05
K27R/N76D/V104Y/N123S/Q206L/N218S	580	0.78	7.40E+05
K27R/M76D/V104Y/N123S/N218S/T260P	660	1	6.60E+05
K27R/N76D/V104Y/N123S/N218S/T274A	590	0.89	6.60E+05
K27R/N76D/V104Y/Q109S/N123S/N218S/T274A	520	1	5.20E+05
K27R/N76D/V104Y/N123S/G195E/D197E/N218S	460	0.65	7.10E+05
B. amyloliquefaciens subtilisin (BPN')	50	0.14	3.60E+05
BFN'-N76D/Y217L*	380	0.46	8.30E+05
* These mutants made as per Example 5, all others made as per Example 2			

Table V

Enzyme	Thermal Stability (% half-life of native enzyme)
B. lentus subtilisin	100

Table V (continued)

Enzyme	Thermal Stability (% half-life of native enzyme)
N76D	590
N76D/S99D	840
N76D/S103A	390
N76D/V104I	660
N76D/I107V	710
N76D/N123S	70
N76D/S99D/S101R	610
N76D/S99D/S103A	590
N76D/S99D/V104I	910
N76D/S101R/S103A	930
N76D/S101R/V104I	500
N76D/S103A/V104I	460
N76D/S103G/V104I*	370
N76D/S103A/V104F	480
N76D/S103A/V104N	230
N76D/S103A/V104S	230
N76D/S103A/V104T	370
N76D/S103A/V104W	280
N76D/S103A/V104Y	400
N76D/V104I/I107V	940
N76D/V104Y/I107V	820
N76D/V104I/N123S	80
N76D/I107V/N123S	150
K27R/N76D/V104Y/N123S	100
N76D/S99D/S101R/S103A	570
N76D/S99D/S101R/V104I	1000
N76D/S99D/S103A/V104I	680
N76D/S101G/S103A/V104I*	390
N76D/S101R/S103A/V104I	470
N76D/S103A/V104I/S105A*	360
N76D/S103A/V104I/S105D*	370
N76D/S103A/V104T/I107A*	270
N76D/S103A/V104T/I107L*	230
N76D/S103A/V104I/N123S	110
N76D/V104I/I107V/N123S	220
N76D/S103A/V104I/L126A	270
N76D/S103A/V104I/L126F	950
N76D/S103A/V104I/L126I	410
N76D/S103A/V104I/L126V	320
N76D/S103A/V104I/S128G*	640
N76D/S103A/VI04I/S128L*	760
N76D/S103A/V104I/L135A	230
N76D/S103A/V104I/L135F	200
N76D/S103A/V104I/L135I	510
N76D/S103A/V104I/L135V	500
N76D/S103A/V104I/S156E*	120
N76D/S103A/V104I/S166D*	590
N76D/S103A/V104I/D197E	460

Table V (continued)

Enzyme	Thermal Stability (% half-life of native enzyme)
N76D/S103A/V104I/N204A*	230
N76D/S103A/V104I/N204G*	240
N76D/S103A/V104I/N204C*	500
N76D/S103A/V104I/P210I*	1370
N76D/S103A/V104I/L217H*	60
N76D/S103A/VI04I/M222A	520
N76D/S103A/VI04I/M222S	490
N76D/S103A/V104I/T260P	490
N76D/S103A/V104I/S265N	360
K27R/N76D/V104Y/I107V/N123S	210
K27R/N76D/V104Y/N123S/D197E	120
K27R/N76D/V104Y/N123S/N204C	110
K27R/N76D/V104Y/N123S/Q206L	380
K27R/N76D/V104Y/N123S/S216V	140
K27R/N76D/V104Y/N123S/N218S	270
K27R1N76D/V104Y/N123S/T260P	40
K27R/N76D/V104Y/N123S/T274A	60
N76D/S99D/S101R/S103A/V104I	590
N76D/S99D/S103A/V104I/N123S	110
N76D/S103A/V104I/L126F/S265N	810
N76D/S103A/V104I/S156E/S166D*	220
K27R/N76D/V104Y/N123S/G195E/G197E	90
K27R/N76D/V104Y/N123S/G195E/N218S	250
K27R/N76D/V104Y/N123S/D197E/N218S	270
K27R/N76D/V104Y/N123S/N204C/N218S	460
K27R/N76D/V104Y/N123S/Q206L/N218S	1400
K27R/N76D/V104Y/N123S/N218S/T260P	310
K27R/N76D/V104Y/N123S/N218S/T274A	180
N76D/S99D/S101R/S103A/V104I/N123S	90
K27R/N76D/V104Y/Q109S/N123S/N218S/T274A	230
K27R/N76D/V104Y/N123S/G195E/D197E/N218S	240
B. amyloliquefaciens subtilisin (BPN')	100
BPN'-N76D/Y217L*	420

* These mutants made as per Example 5, all others made as per Example 2

Example 6

Wash Performance Test

[0065] The wash performance of the variants described in the previous examples was evaluated by measuring the removal of stain from EMPA 116 (blood/milk/carbon black on cotton) cloth swatches (Testfabrics, Inc., Middlesex, NJ 07030).

[0066] Six EMPA 116 swatches, cut to 3 X 4-1/2 inches with pinked edges, were placed in each pot of a Model 72435 Terg-O-Tometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 15 gpg hardness (Ca^{++}: Mg^{++}::3:1::w:w), 7 g of detergent, and enzyme as appropriate. The detergent base was WFK1 detergent from wfk - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany:

Component	% of Final Formulation
Zeolite A	25%
Sodium sulfate	25%
Soda Ash	10%
Linear alkylbenzenesulfonate	8.8%
Alcohol ethoxylate (7-8 EO)	4.5%
Sodium soap	3%
Sodium silicate (SiO_2:Na_2O::3.3:1)	3%

[0067] To this base detergent, the following additions were made:

Component	% of Final Formulation
Sodium perborate monohydrate	13%
Copolymer (Sokalan CP5)	4%
TAED (Mykon ATC Green)	3%
Enzyme	0.5%
Brightener (Tinopal AMS-GX)	0.2%

[0068] Sodium perborate monohydrate was obtained from Degussa Corporation, Ridgefield-Park,_NJ 07660. Sokalan CP5 was obtained from BASF Corporation, Parsippany, NJ 07054. Mykon ATC Green (TAED, tetraacetylethylenediamine) was obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England. Tinopal AMS GX was obtained from Ciba-Geigy Corporation, Greensboro, NC 27419.

[0069] Six EMPA 116 swatches were washed in detergent with enzyme for 30 minutes at 60°C and were subsequently rinsed twice for 5 minutes each time in 1000 ml water. Enzymes were added at final concentrations of 0.05 to 1 ppm for standard curves, and 0.25 ppm for routine analyses. Swatches were dried and pressed, and the reflectance from the swatches was measured using the L value on the L*a*b* scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as a percentage of the performance of *B. lentus* (GG36) protease and was calculated by dividing the amount of *B. lentus* (GG36) protease by the amount of variant protease that was needed to provide the same stain removal performance X 100. The data are shown in Table VII.

Table VI

Enzyme	**Wash Performance**
B. lentus subtilisin	100
N76D	310
N76D/S103A	230
N76D/V104I	130
N76D/I107V	160
N76D/S99D/S101R	370
N76D/S99D/S103A	290
N76D/S101R/S103A	130
N76D/S101R/V104I	300
N76D/S103A/V104I	320
N76D/S103G/V104I	160
N76D/S103A/V104F	210
N76D/S103A/V109N	110
N76D/S103A/V104T	170
N76D/V104I/I107V	210
N76D/S99D/S101R/S103A	220
N76D/S99D/S101R/V104I	140

Table VI (continued)

Enzyme	Wash Performance
N76D/S101G/S103A/V104I	170
N76D/S101R/S103A/V104I	150
N76D/S103A/V104I/S105A	170
N76D/S103A/V104T/I107A	120
N76D/S103A/V104T/I107L	110
N76D/S103A/V104I/L126F	110
N76D/S103A/V104I/S128G	280
N76D/S103A/V104I/L135I	160
N76D/S103A/V104I/L135V	160
N76D/S103A/V104I/D197E	170
N76D/S103A/V104I/N204A	160
N76D/S103A/V104I/N204G	150
N76D/S103A/V104I/P210I	470
N76D/S103A/V104I/M222A	100
N76D/S103A/V104I/T260P	280
N76D/S103A/V104I/S265N	190

Example 7

Protease Stability in a Liquid Detergent Formulation

[0070] A comparison of protease stability toward inactivation in a liquid detergent formulation was made for *Bacillus lentus* subtilisin and it's variant enzyme N76D/S103A/V104I according to the procedure outlined herein. The detergent formulation used for the study was a commercially purchased bottle of Tide Ultra liquid lanudry detergent made in the USA by Procter & Gamble Company. Heat treatment of the detergent formulation was necessary to inactivate in-situ protease. This was accomplished by incubating the detergent at 96°C for a period of 4.5 hours. Concentrated preparations of the *B. lentus* subtilisin and N76D/S103A/V104I variant, in the range of 20 grams/liter enzyme, were then added to the heat-treated Tide Ultra at room-temperature to a final concentratrion of 0.3 grams/liter enzyme in the detergent formulation. The heat-treated detergent with protease added was then incubated in a water bath thermostatted at 50°C. Aliquots were removed from the incubation tubes at 0, 24, 46, 76, and 112 hour time intervals and assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate suc-Ala-Ala-Pro-phe-p-nitroanilide dissolved in 0.1M tris-HCL buffer, pH 8.6, and thermostatted at 25°C. The initial linear reaction velocity was followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410nm as a function of time. As shown in Fig. 10, the N76D/S103A/V104I variant was observed to have significantly greater stability towards inactivation than the native *B. lentus* enzyme. Estimated half-lives for inactivation in the Tide Ultra detergent formulation for the two enzymes, under the specified test conditions, are 45 hours for B. lentus subtilisin and 125 hours for the N76D/S103A/V104I variant.

[0071] Throughout this application reference is made to various amino acids by way of common one- and three-letter codes. Such codes are identified in Dale, J.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

[0072] Although the preferred embodiments of the invention have been described above, it will be obvious to those skilled in the art to which the invention pertains, that, after understanding the invention as a whole, various changes and equivalent modifications may be made without departing from the scope of the invention as defined by the appended claims.

SEQUENCE LISTING

[0073]

(1) GENERAL INFORMATION:

(i) APPLICANT: Graycar, Thomas P

Bott, Richard R
Wilson, Lori J

(ii) TITLE OF INVENTION: Subtilisin Variants

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: 180 Kimball Way
(C) CITY: So. San Francisco
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 13-OCT-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Horn, Margaret A.
(B) REGISTRATION NUMBER: 33,401
(C) REFERENCE/DOCKET NUMBER: GC235-2

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 742-7536
(B) TELEFAX: (415) 742-7217

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGCTGCAA CTCGTTAAA 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGCTCTAG ACAATTCG 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATTAGGGG CGGACGGTCG AGGCGCCATC AGCTCGATT 39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAGGTTCGG TCTCGAGCGT TGCCCAAGGA TTG 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACGTTGCTA GCTTGAGTTT AG 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1497 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCTACTAA AATATTATTC CATACTATAC AATTAATACA CAGAATAATC TGTCTATTGG 60
TTATTCTGCA AATGAAAAAA AGGAGAGGAT AAAGAGTGAG AGGCAAAAAA GTATGGATCA 120
GTTTGCTGTT TGCTTTAGCG TTAATCTTTA CGATGGCGTT CGGCAGCACA TCCTCTGCCC 180
AGGCGGCAGG GAAATCAAAC GGGGAAAAGA AATATATTGT CGGGTTTAAA CAGACAATGA 240
GCACGATGAG CGCCGCTAAG AAGAAAGATG TCATTTCTGA AAAAGGCGGG AAAGTGCAAA 300
AGCAATTCAA ATATGTAGAC GCAGCTTCAG TCACATTAAA CGAAAAAGCT GTAAAAGAAT 360

TGAAAAAAGA CCCGAGCGTC GCTTACGTTG AAGAAGATCA CGTAGCACAT GCGTACGCGC 420
AGTCCGTGCC TTACGGCGTA TCACAAATTA AAGCCCCTGC TCTGCACTCT CAAGGCTACA 480
CTGGATCAAA TGTTAAAGTA GCGGTTATCG ACAGCGGTAT CGATTCTTCT CATCCTGATT 540
TAAAGGTAGC AAGCGGAGCC AGCATGGTTC CTTCTGAAAC AAATCCTTTC CAAGACAACA 600
ACTCTCACGG AACTCACGTT GCCGGCACAG TTGCGGCTCT TAATAACTCA ATCGGTGTAT 660
TAGGCGTTGC GCCAAGCGCA TCACTTTACG CTGTAAAAGT TCTCGGTGCT GACGGTTCCG 720
GCCAATACAG CTGGATCATT AACGGAATCG AGTGGGCGAT CGCAAACAAT ATGGACGTTA 780
TTAACATGAG CCTCGGCGGA CCTTCTGGTT CTGCTGCTTT AAAAGCGGCA GTTGATAAAG 840
CCGTTGCATC CGGCGTCGTA GTCGTTGCGG CAGCCGGTAA CGAAGGCACT TCCGGCAGCT 900
CAAGCACAGT GGGCTACCCT GGTAAATACC CTTCTGTCAT TGCAGTAGGC GCTGTTGACA 960
GCAGCAACCA AAGAGCATCT TTCTCAAGCG TAGGACCTGA GCTTGATGTC ATGGCACCTG 1020
GCGTATCTAT CCAAAGCACG CTTCCTGGAA ACAAATACGG GGCGTACAAC GGTACGTCAA 1080
TGGCATCTCC GCACGTTGCC GGAGCGGCTG CTTTGATTCT TTCTAAGCAC CCGAACTGGA 1140
CAAACACTCA AGTCCGCAGC AGTTTAGAAA ACACCACTAC AAAACTTGGT GATTCTTTGT 1200
ACTATGGAAA AGGGCTGATC AACGTACAAG CGGCAGCTCA GTAAAACATA AAAAACCGGC 1260
CTTGGCCCCG CCGGTTTTTT ATTATTTTTC TTCCTCCGCA TGTTCAATCC GCTCCATAAT 1320
CGACGGATGG CTCCCTCTGA AAATTTTAAC GAGAAACGGC GGGTTGACCC GGCTCAGTCC 1380
CGTAACGGCC AACTCCTGAA ACGTCTCAAT CGCCGCTTCC CGGTTTCCGG TCAGCTCAAT 1440
GCCATAACGG TCGGCGGCGT TTTCCTGATA CCGGGAGACG GCATTCGTAA TCGGATC 1497

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
1               5                  10                  15

His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
            20                  25                  30

Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
        35                  40                  45

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
    50                  55                  60

Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
65                  70                  75                  80

Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
                85                  90                  95

Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
            100                 105                 110

Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
        115                 120                 125

Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
    130                 135                 140

Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
145                 150                 155                 160

Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
                165                 170                 175

Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
            180                 185                 190

Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
        195                 200                 205

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
    210                 215                 220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225                 230                 235                 240

Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
                245                 250                 255

Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
            260                 265                 270

Ala Ala Gln
        275
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:B:

```
Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu
1               5                  10                  15

His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
            20                  25                  30

Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala
        35                  40                  45

Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His
    50                  55                  60

Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly
65                  70                  75                  80

Val Leu Gly Val Ser Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
                85                  90                  95

Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
            100                 105                 110

Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
        115                 120                 125

Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser
    130                 135                 140

Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly
145                 150                 155                 160

Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala
                165                 170                 175

Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala
            180                 185                 190

Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
        195                 200                 205

Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr
    210                 215                 220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr
225                 230                 235                 240

Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr
                245                 250                 255

Leu Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
            260                 265                 270

Ala Ala Gln
        275
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 274 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys Val
1               5                  10                  15

Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala Val Leu Asp
            20                  25                  30

Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val Gly Gly Ala
        35                  40                  45

Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn Gly His Gly
    50                  55                  60

Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr Thr Gly Val
65                  70                  75                  80

Leu Gly Val Ala Pro Ser Val Ser Leu Tyr Ala Val Lys Val Leu Asn
                85                  90                  95

Ser Ser Gly Ser Gly Ser Tyr Ser Gly Ile Val Ser Gly Ile Glu Trp
            100                 105                 110

Ala Thr Thr Asn Gly Met Asp Val Ile Asn Met Ser Leu Gly Gly Ala
        115                 120                 125

Ser Gly Ser Thr Ala Met Lys Gln Ala Val Asp Asn Ala Tyr Ala Arg
    130                 135                 140

Gly Val Val Val Val Ala Ala Ala Gly Asn Ser Gly Asn Ser Gly Ser
145                 150                 155                 160

Thr Asn Thr Ile Gly Tyr Pro Ala Lys Tyr Asp Ser Val Ile Ala Val
                165                 170                 175

Gly Ala Val Asp Ser Asn Ser Asn Arg Ala Ser Phe Ser Ser Val Gly
            180                 185                 190

Ala Glu Leu Glu Val Met Ala Pro Gly Ala Gly Val Tyr Ser Thr Tyr
        195                 200                 205

Pro Thr Asn Thr Tyr Ala Thr Leu Asn Gly Thr Ser Met Ala Ser Pro
    210                 215                 220

His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Leu
225                 230                 235                 240

Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala Thr Tyr Leu
                245                 250                 255

Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Glu Ala Ala
            260                 265                 270

Ala Gln
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 269 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala
1               5                  10                  15

His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp
            20                  25                  30

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45

Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80

Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95

Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125

Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140

Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
145                 150                 155                 160

Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175

Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190

Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205

Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220

Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240

Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255

Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1140 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTTT    60
AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT   120
GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT   180
CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT   240
TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTTCT   300
TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAATTAGC   360
CGTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT   420
GTCCTCGATA CAGGTATTTC CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT   480
GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG   540
ATTGCTGCTT TAAACAATTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACTATAC   600
GCTGTTAAAG TATTAGGGGC GAGCGGTTCA GGTTCGGTCA GCTCGATTGC CCAAGGATTG   660
GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTTGA GTTTAGGAAG CCCTTCGCCA   720
AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG   780
GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG   840
GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG   900
CTTGACATTG TCGCACCAGG TGTAAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC   960
AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA  1020
CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG  1080
AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC  1140
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1140 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTTT    60
AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT   120
GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT   180
CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT   240
TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTTCT   300
TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAATTAGC   360
CGTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT   420
GTCCTCGATA CAGGTATTTC CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT   480
GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG   540
ATTGCTGCTT TAGACAACTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACTATAC   600
GCTGTTAAAG TATTAGGGGC GAGCGGTTCA GGCGCCATCA GCTCGATTGC CCAAGGATTG   660
GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTTGA GTTTAGGAAG CCCTTCGCCA   720
AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG   780
GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG   840
GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG   900
CTTGACATTG TCGCACCAGG TGTAAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC   960
AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA  1020
CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG  1080
AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC  1140
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
TATGCCAGCC ACAACGGTAC TTCGATGGCT                                     30
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGTTGCG GCTCTAGATA ACTCAATCGG T 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGACGGTT CCGGCGCTAT TAGTTGGATC ATT 33

Claims

1. A subtilisin variant having an amino acid sequence not found in nature, derived from a precursor subtilisin, comprising substitutions corresponding to: N76D/S103A; N76D/V104I; N76D/I107V or N76D/S99D/S101R of *B. amyloliquefaciens* subtilisin, wherein the subtilisin variant has improved wash performance as compared to the precursor subtilisin.

2. A subtilisin variant of claim 1 wherein the subtilisin variants comprise substitutions corresponding to: N76D/S99D/S103A; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/S103G/V104I; N76D/S103A/V104F; N76D/S103A/V104N; N76D/S103A/V104T; N76D/V104I/I107V; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S101G/S103A/V104I; N76D/S101R/S103A/V109I; N76D/S103A/V104I/S105A; N76D/S103A/V104T/I107A; N76D/S103A/V104T/I107L; N76D/S103A/V104I/L126F; N76D/S103A/V104I/S128G; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L135V; N76D/S103A/V104I/D197E; N76D/S103A/V104I/N204A; N76D/S103A/V104I/N204G; N76D/S103A/V104I/P210I; N76D/S103A/V104I/T260P or N76D/S103A/V104I/S265N of *B. amyloliquefaciens* subtilisin.

3. A subtilisin variant according to claim 1 or claim 2 wherein the precursor subtilisin is a Bacillus subtilisin.

4. A subtilisin variant according to claim 3 wherein the precursor subtilisin is *Bacillus lentus* subtilisin.

5. DNA encoding a subtilisin variant of claim 1 or claim 2.

6. An expression vector encoding the DNA of claim 5.

7. A host cell transformed with the expression vector of claim 6.

Patentansprüche

1. Subtilisinvariante mit einer nicht in der Natur vorkommenden Aminosäuresequenz, die von einem Vorläufersubtilisin stammt, das Substitutionen umfasst, die N76D/S103A; N76DN1041; N76D/I107V oder N76D/S99D/S101R von B.-amyloliquefaciens-Subtilisin entsprechen, worin die Subtilisinvariante im Vergleich zum Vorläufersubtilisin verbesserte Waschleistung aufweist.

2. Subtilisinvariante nach Anspruch 1, worin die Subtilisinvarianten Substitutionen umfassen, die
N76D/S99D/SI03A; N76D/S101R/S103A; N76D/S101R/V104I;
N76D/S103A/V104I; N76D/S103G/V104I; N76D/S103A/V104F;

N76D/S103A/V104N; N76D/S103A/V104T; N76D/V104I/I107V;
N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I;
N76D/S101G/S103A/V104I; N76D/S101R/S103A/V104I;
N76D/S103A/V104I/S105A; N76D/S103A/V104T/I107A;
N76D/S103A/V104T/I107L; N76D/S103A/V104I/L126F;
N76D/S103A/V104I/S128G; N76D/S103A/V104I/L135I;
N76D/S103A/V104I/L135V; N76D/S103A/V104I/D197E;
N76D/S103A/V109I/N204A; N76D/S103A/V104I/N204G;
N76D/S103A/V104I/P210I; N76D/S103A/V104I/T260P oder
N76D/S103A/V104I/S265N
von B.-amyloliquefaciens-Subtilisin entsprechen.

3. Subtilisinvariante nach Anspruch 1 oder Anspruch 2, worin das Vorläufersubtilisin ein Bacillus-Subtilisin ist.

4. Subtilisinvariante nach Anspruch 3, worin das Vorläufersubtilisin Bacillus-lentus-Subtilisin ist.

5. DNA, die für eine Subtilisinvariante nach Anspruch 1 oder Anspruch 2 kodiert.

6. Expressionsvektor, der für die DNA nach Anspruch 5 kodiert.

7. Wirtszelle, die mit dem Expressionsvektor nach Anspruch 6 transformiert ist.

Revendications

1. Variant de subtilisine ayant une séquence d'acides aminés que l'on ne trouve pas dans la nature, dérivé d'une subtilisine précurseur, comprenant des substitutions correspondant à: N76D/S103A; N76D/V104I; N76D/I107V ou N76D/S99D/S101R de la subtilisine de *B. amyloliquefaciens*, où le variant de subtilisine a une meilleure performance de lavage en comparaison avec la subtilisine précurseur.

2. Variant de subtilisine de la revendication 1 où les variants de subtilisine comprennent des substitu tions correspondant à:

N76D/S99D/S103A; N76D/S101R/S103A; N76D/S101R/V104I;
N76D/S103A/V104I; N76D/S103G/V104I; N76D/S103A/V104F;
N76D/S103A/V104N; N76D/S103A/V104T; N76D/V104I; I107V;
N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I;
N76D/S101G/S103A/V104I; N76D/S101R/S103A/V104I;
N76D/S103A/V104I/S105A; N76D/S103A/V104T/I107A;
N76D/S103A/V104T/I107L; N76D/S103A/V104I/L126F;
N76D/S103A/V104I/S128G; N76D/S103A/V104I/L135I;
N76D/S103A/V104I/L135V; N76D/S103A/V104I/D197E;
N76D/S103A/V104I/N204A; N76D/S103A/V104I/N204G;
N76D/S103A/V104I/P210I; N76D/S103A/V104I/T260P ou
N76D/S103A/V104I/S265N de la subtilisine du B. amyloliquefaciens.

3. Variant de subtilisine selon la revendication 1 ou la revendication 2 où la subtilisine précurseur est une substilisine de *Bacillus*.

4. Variant de subtilisine selon la revendication 3 où la subtilisine précurseur est la subtilisine de *Baccilus lentus*.

5. ADN codant pour un variant de subtilisine de la revendication 1 ou de la revendication 2.

6. Vecteur d'expression codant pour l'ADN de la revendication 5.

7. Cellule hôte transformée par le vecteur d'expression de la revendication 6.

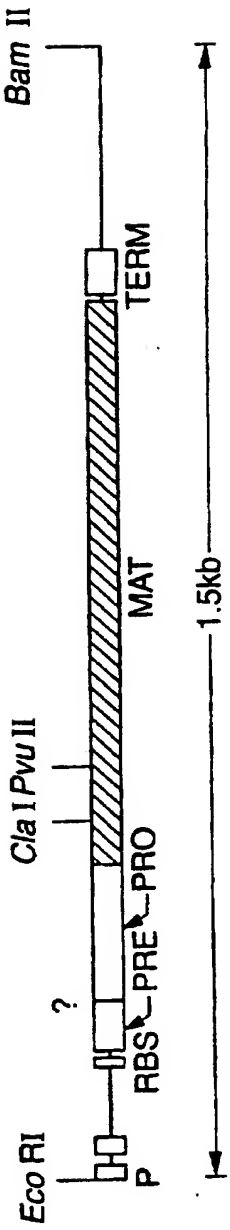

FIG._1A

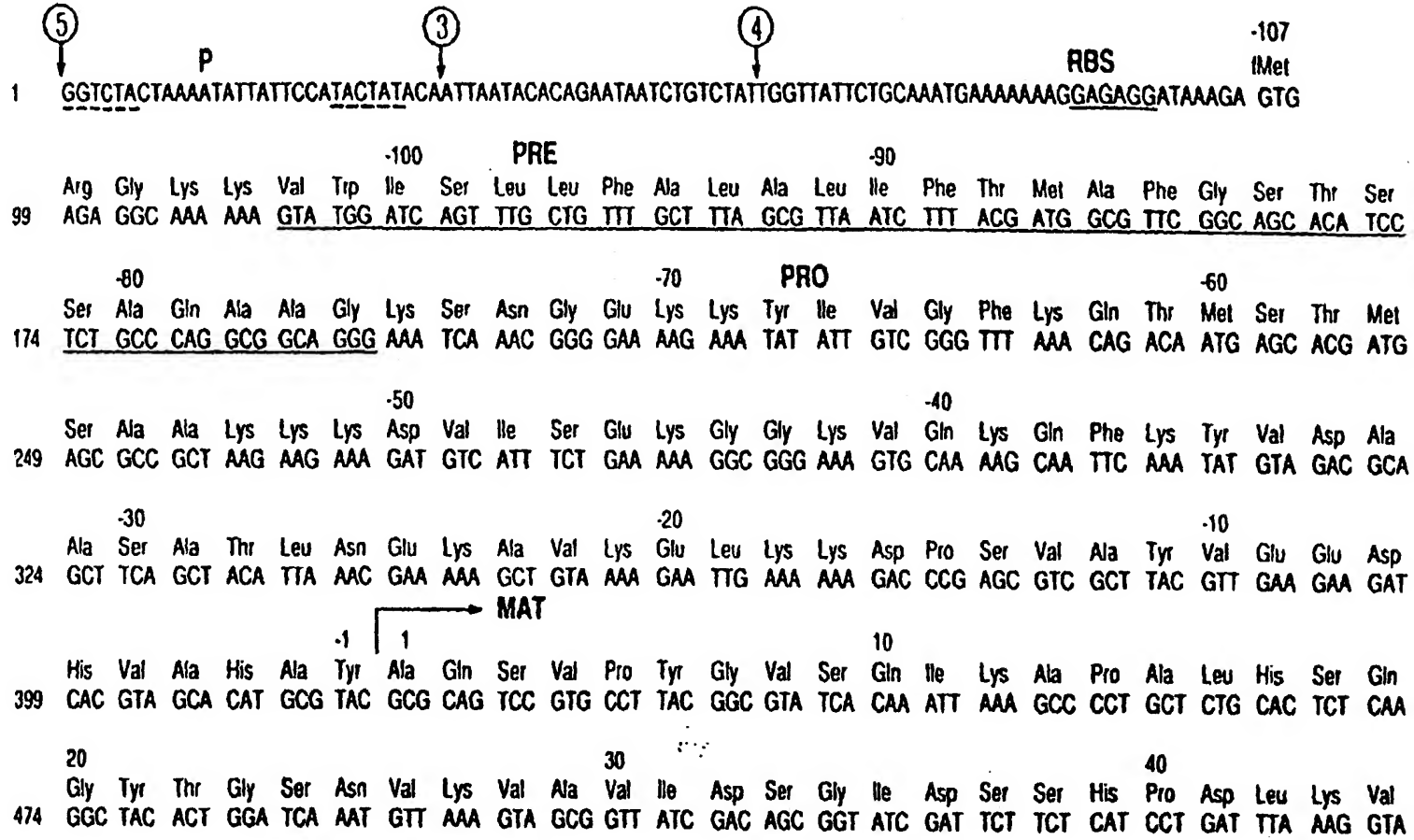

FIG._1B - 1

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						50						Pro	Asn			60	Asp							
	Ala	Gly	Gly	Ala	Ser	Met	Val	Pro	Ser	Glu	Thr	Asn	Pro	Phe	Gln	Asp	Asn	Asn	Ser	His	Gly	Thr	His	Val	Ala
549	GCA	GGC	GGA	GCC	AGC	ATG	GTT	CCT	TCT	GAA	ACA	AAT	CCT	TTC	CAA	GAC	AAC	AAC	TCT	CAC	GGA	ACT	CAC	GTT	GCC

	70										80								Ser	Ala	90				
	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys
624	GGC	ACA	GTT	GCG	GCT	CTT	AAT	AAC	TCA	ATC	GGT	GTA	TTA	GGC	GTT	GCG	CCA	AGC	GCA	TCA	CTT	TAC	GCT	GTA	AAA

				Asp	Ala	100										110									
	Val	Leu	Gly	Ala	Asp	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	Trp	Ala	Ile	Ala	Asn	Asn	Met
699	GTT	CTC	GGT	GCT	GAC	GGT	TCC	GGC	CAA	TAC	AGC	TGG	ATC	ATT	AAC	GGA	ATC	GAG	TGG	GCG	ATC	GCA	AAC	AAT	ATG

	120										130									140					
	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Pro	Ser	Gly	Ser	Ala	Ala	Leu	Lys	Ala	Ala	Val	Asp	Lys	Ala	Val	Ala
774	GAC	GTT	ATT	AAC	ATG	AGC	CTC	GGC	GGA	CCT	TCT	GGT	TCT	GCT	GCT	TTA	AAA	GCG	GCA	GTT	GAT	AAA	GCC	GTT	GCA

						150								Ser	Thr	160									
	Ser	Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Glu	Gly	Thr	Ser	Gly	Ser	Ser	Ser	Thr	Val	Gly	Tyr	Pro	Gly
849	TCC	GGC	GTC	GTA	GTC	GTT	GCG	GCA	GCC	GGT	AAC	GAA	GGC	ACT	TCC	GGC	AGC	TCA	AGC	ACA	GTG	GGC	TAC	CCT	GGT

	170										180									190					
	Lys	Tyr	Pro	Ser	Val	Ile	Ala	Val	Gly	Ala	Val	Asp	Ser	Ser	Asn	Gln	Arg	Ala	Ser	Phe	Ser	Ser	Val	Gly	Pro
924	AAA	TAC	CCT	TCT	GTC	ATT	GCA	GTA	GGC	GCT	GTT	GAC	AGC	AGC	AAC	CAA	AGA	GCA	TCT	TTC	TCA	AGC	GTA	GGA	CCT

						200										210									
	Glu	Leu	Asp	Val	Met	Ala	Pro	Gly	Val	Ser	Ile	Gln	Ser	Thr	Leu	Pro	Gly	Asn	Lys	Tyr	Gly	Ala	Tyr	Asn	Gly
999	GAG	CTT	GAT	GTC	ATG	GCA	CCT	GGC	GTA	TCT	ATC	CAA	AGC	ACG	CTT	CCT	GGA	AAC	AAA	TAC	GGG	GCG	TAC	AAC	GGT

	220										230									240					
	Thr	Ser	Met	Ala	Ser	Pro	His	Val	Ala	Gly	Ala	Ala	Ala	Leu	Ile	Leu	Ser	Lys	His	Pro	Asn	Trp	Thr	Asn	Thr
1074	ACG	TCA	ATG	GCA	TCT	CCG	CAC	GTT	GCC	GGA	GCG	GCT	GCT	TTG	ATT	CTT	TCT	AAG	CAC	CCG	AAC	TGG	ACA	AAC	ACT

FIG._1B - 2

```
                                    250 Gln                                                 260
     Gln  Val  Arg  Ser  Ser  Leu  Glu  Asn  Thr  Thr  Thr  Lys  Leu  Gly  Asp  Ser  Phe  Tyr  Tyr  Gly  Lys  Gly  Leu  Ile  Asn
1149 CAA  GTC  CGC  AGC  AGT  TTA  GAA  AAC  ACC  ACT  ACA  AAA  CTT  GGT  GAT  TCT  TTC  TAC  TAT  GGA  AAA  GGG  CTG  ATC  AAC

     270                       275
     Val  Gln  Ala  Ala  Ala  Gln  OC                    TERM
1224 GTA  CAG  GCG  GCA  GCT  CAG  TAA  AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC
```

FIG._1B - 3

FIG._1B

- *FIG._1B - 1*
- *FIG._1B - 2*
- *FIG._1B - 3*

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CONSERVED RESIDUES IN SUBTILISINS FROM *BACILLUS AMYLOLIQUEFACIENS*

```
1                   10                  20
A Q S V P . G . . . . . A P A . H . . G

21                  30                  40
. T G S . V K V A V . D . G . . . . H P

41                  50                  60
D L . . . G G A S . V P . . . . . Q D

61                  70                  80
. N . H G T H V A G T . A A L N N S I G

81                  90                  100
V L G V A P S A . L Y A V K V L G A . G

101                 110                 120
S G . . S . L . . G . E W A . N . . . .

121                 130                 140
V . N . S L G . P S . S . . . . . A . .

141                 150                 160
. . . . . G V . V V A A . G N . G . . .

161                 170                 180
. . . . . Y P . . Y . . . . A V G A .

181                 190                 200
D . . N . . A S F S . . G . . L D . . A

201                 210                 220
P G V . . Q S T . P G . . Y . . . N G T

221                 230                 240
S M A . P H V A G A A A L . . . K . . .

241                 250                 260
W . . . Q . R . . L . N T . . . L G . .

261                 270
. . Y G . G L . N . . A A . .
```

FIG._2

COMPARISON OF SUBTILISIN SEQUENCES FROM:

B.amyloliquefaciens
B.subtilis
B.licheniformis
B.lentus

```
01                  10                  20                  30
A Q S V P Y G V S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q T V P Y G I P L I K A D K V Q A Q G F K G A N V K V A V L D T G I Q A S H P
A Q S V P W G I S R V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P

41                  50                  60                  70
D L K V A G G A S M V P S E T N P F Q D N N S H G T H V A G T V A A L N N S I G
D L N V R G G A S F V P S E T N P Y Q D G S S H G T H V A G T I A A L N N S I G
D L N V V G G A S F V A G E A Y N * T D G N G H G T H V A G T V A A L D N T T G
D L N I R G G A S F V P G E * P S T Q D G N G H G T H V A G T I A A L N N S I G

81                  90                  100                 110
V L G V A P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I A N N M D
V L G V S P S A S L Y A V K V L D S T G S G Q Y S W I I N G I E W A I S N N M D
V L G V A P S V S L Y A V K V L N S S G S G S Y S G I V S G I E W A T T N G M D
V L G V A P S A E L Y A V K V L G A S G S G S V S S I A Q G L E W A G N N G M H

121                 130                 140                 150
V I N M S L G G P S G S A A L K A A V D K A V A S G V V V V A A A G N E G T S G
V I N M S L G G P T G S T A L K T V V D K A V S S G I V V A A A A G N E G S S G
V I N M S L G G A S G S T A M K Q A V D N A Y A R G V V V V A A A G N S G N S G
V A N L S L G S P S P S A T L E Q A V N S A T S R G V L V V A A S G N S G A G S
```

FIG._3A

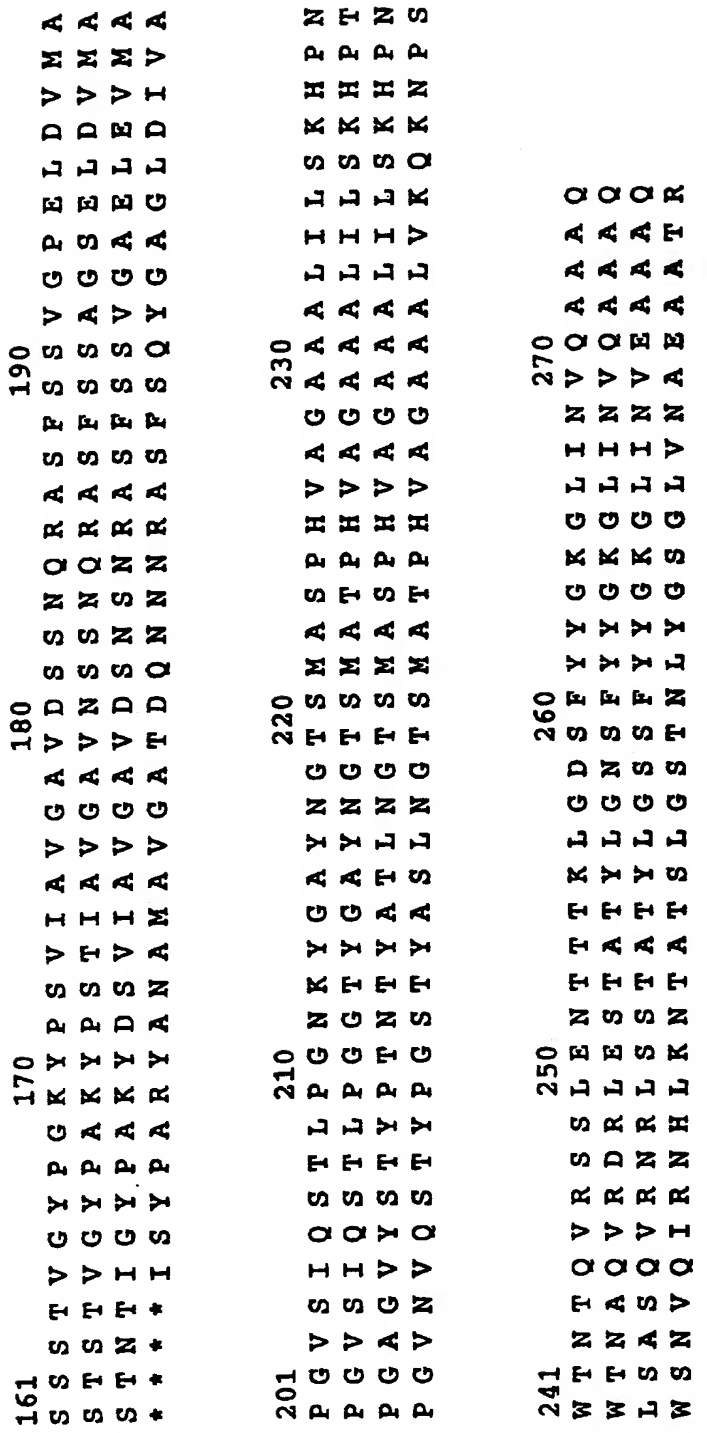
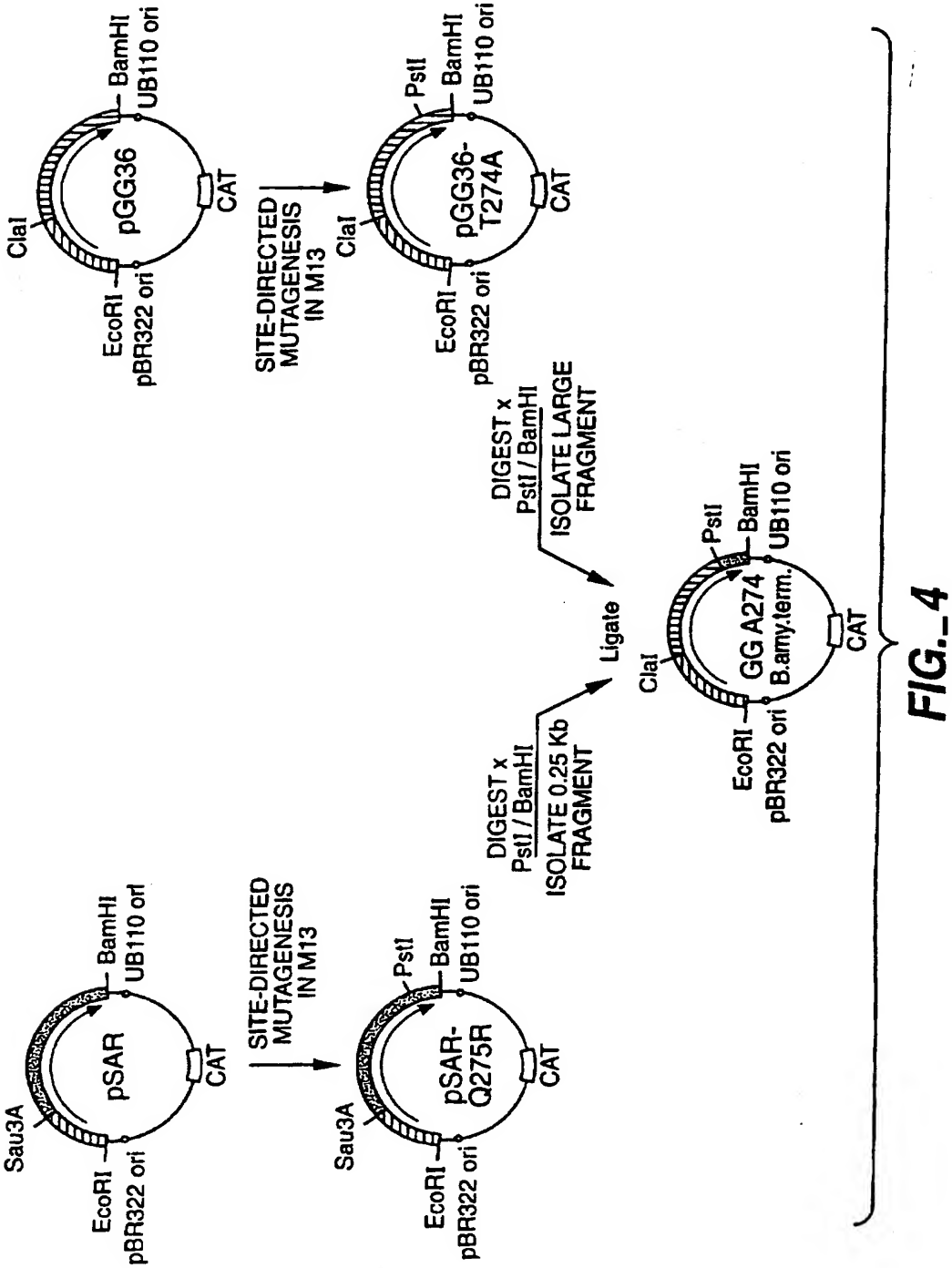


FIG._3B

FIG._3

FIG._3A

FIG._3B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BamHI
UB110 ori
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MUTAGENESIS
IN M13
PstI
pGG36-
T274A
DIGEST x
PstI / BamHI
ISOLATE LARGE
FRAGMENT
Sau3A
pSAR
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PstI / BamHI
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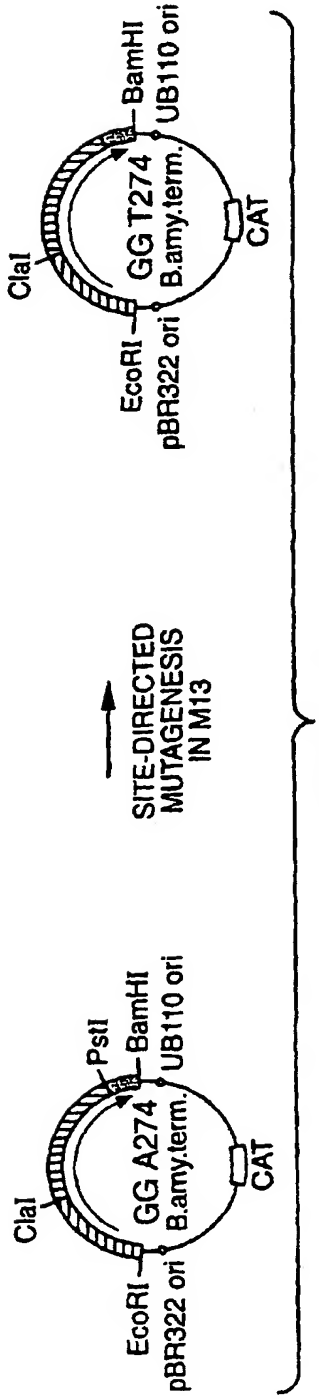
FIG._4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CAT
SITE-DIRECTED
MUTAGENESIS
IN M13
ClaI
EcoRI
pBR322 ori
GG T274
B.amy.term.
BamHI
UB110 ori
CAT

FIG._5

```
        10                  30                  50
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

        70                  90                  110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

       130                 150                 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

       190                 210                 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

       250                 270                 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

       310                 330                 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

       370                 390                 410
CGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla
```

FIG._6A

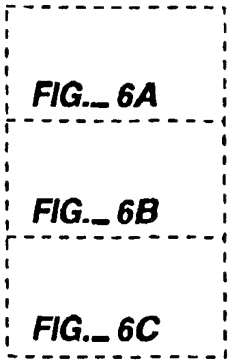
```
       430                   450                   470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

       490                   510                   530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

       550                   570                   590
ATTGCTGCTTTAAACAATTCGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACTATAC
IleAlaAlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

       610                   630                   650
GCTGTTAAAGTATTAGGGGCGAGCGGTTCAGGTTCGGTCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeu

       670                   690                   710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro

       730                   750                   770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

       790                   810                   830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet
```

FIG._6B

```
       850                  870                  890
GCAGTCGGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly

       910                  930                  950
CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla

       970                  990                 1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys

      1030                 1050                 1070
CAAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr

      1090                 1110                 1130
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg
```

FIG._6C

FIG._6



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```
        10                  30                  50
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

        70                  90                  110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

       130                 150                 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

       190                 210                 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

       250                 270                 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

       310                 330                 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

       370                 390                 410
CGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla
```

FIG._7A

```
        430                   450                   470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

        490                   510                   530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

        550                   570                   590
ATTGCTGCTTTAGACAACTCGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACTATAC
IleAlaAlaLeuAspAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

        610                   630                   650
GCTGTTAAAGTATTAGGGGCGAGCGGTTCAGGCGCCATCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlyAlaIleSerSerIleAlaGlnGlyLeu

        670                   690                   710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro

        730                   750                   770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

        790                   810                   830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet
```

FIG._7B

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```
       850                   870                   890
GCAGTCGGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly

       910                   930                   950
CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla

       970                   990                  1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys

      1030                  1050                  1070
CAAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr

      1090                  1110                  1130
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg
```

FIG._7C

FIG._7

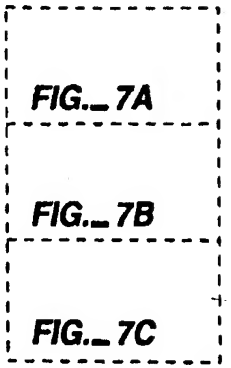

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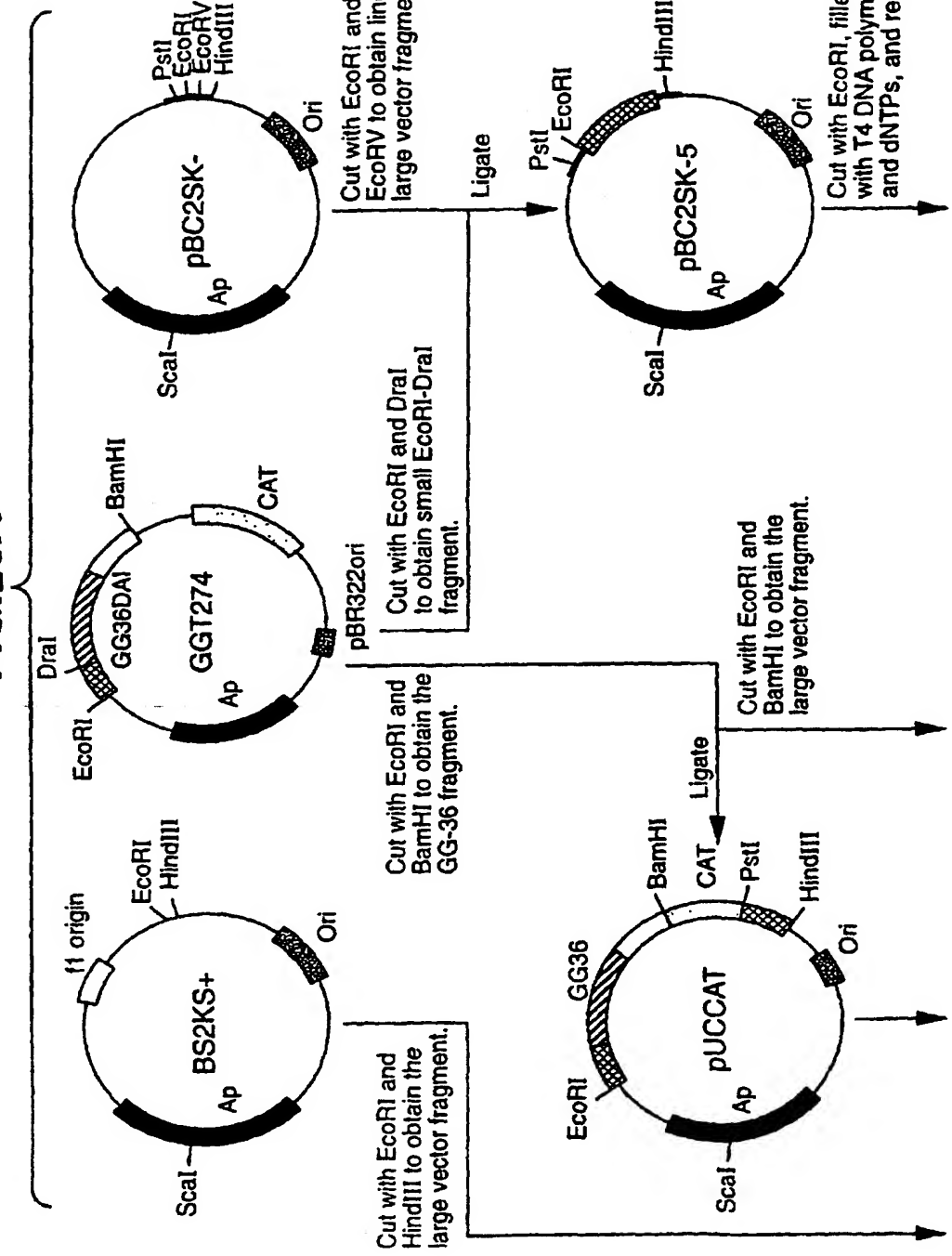
FIG._8A


f1 origin
EcoRI
HindIII
BS2KS+
Ap
Ori
ScaI
Cut with EcoRI and HindIII to obtain the large vector fragment.
DraI
EcoRI
GG36DAI
BamHI
GGT274
CAT
Ap
pBR322ori
Cut with EcoRI and BamHI to obtain the GG-36 fragment.
PstI
EcoRI
EcoRV
HindIII
pBC2SK-
Ap
Ori
ScaI
Cut with EcoRI and EcoRV to obtain linear large vector fragment.
Ligate
PstI
EcoRI
HindIII
pBC2SK-5
Ap
Ori
ScaI
Cut with EcoRI, filled in with T4 DNA polymerase and dNTPs, and religate.
Cut with EcoRI and BamHI to obtain the large vector fragment.
Ligate
EcoRI
GG36
BamHI
CAT
PstI
HindIII
pUCCAT
Ap
Ori
ScaI

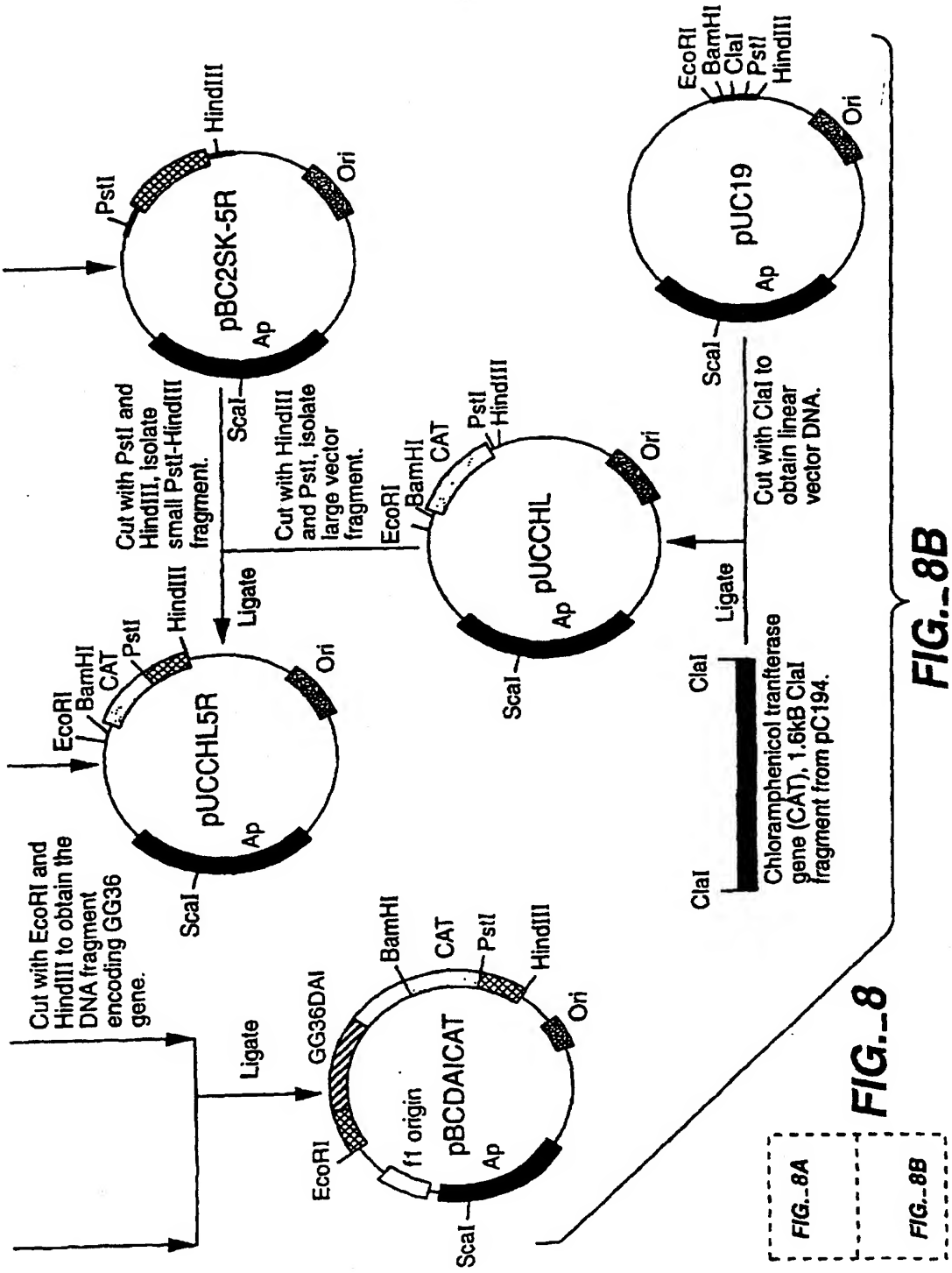
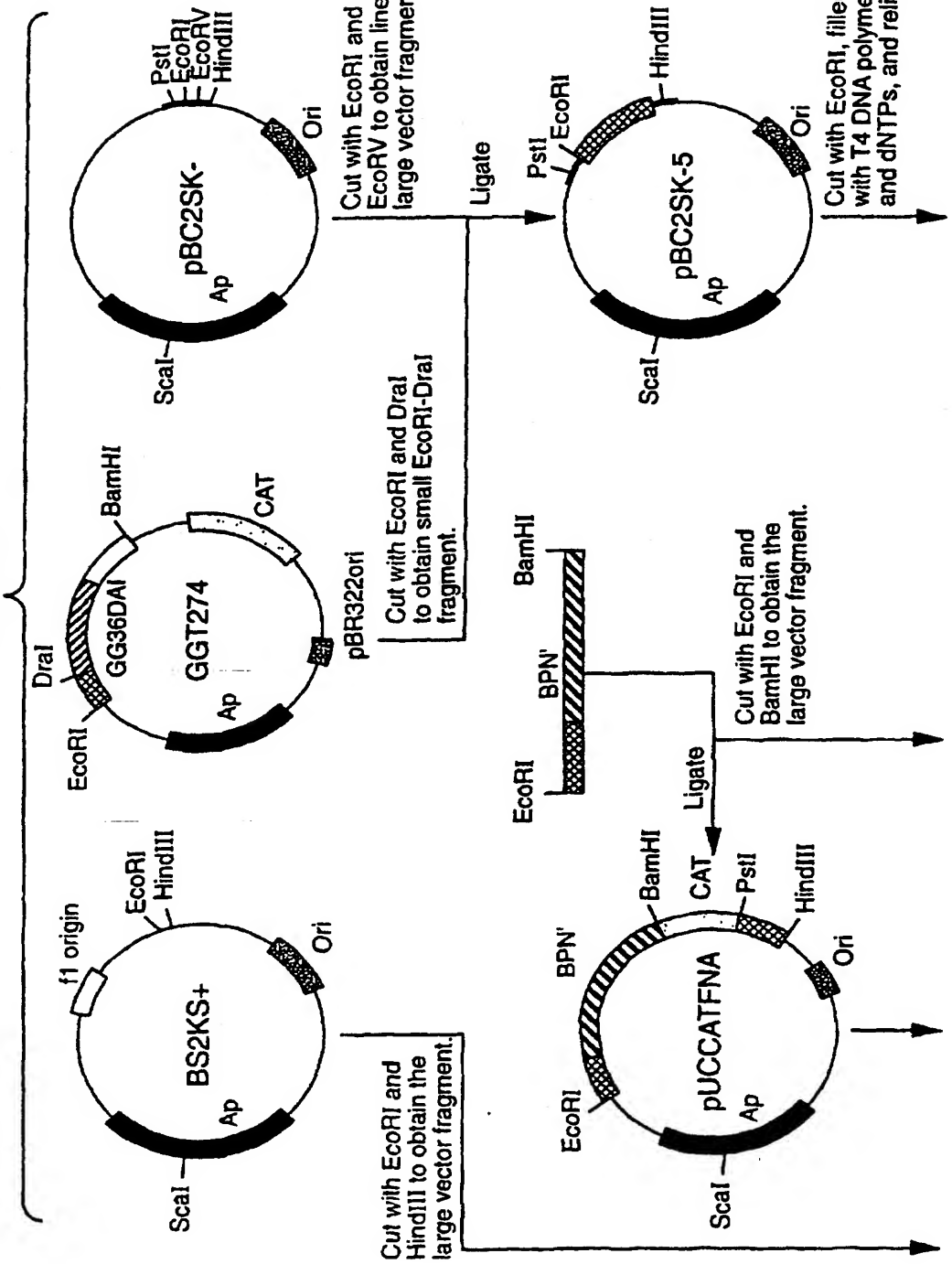
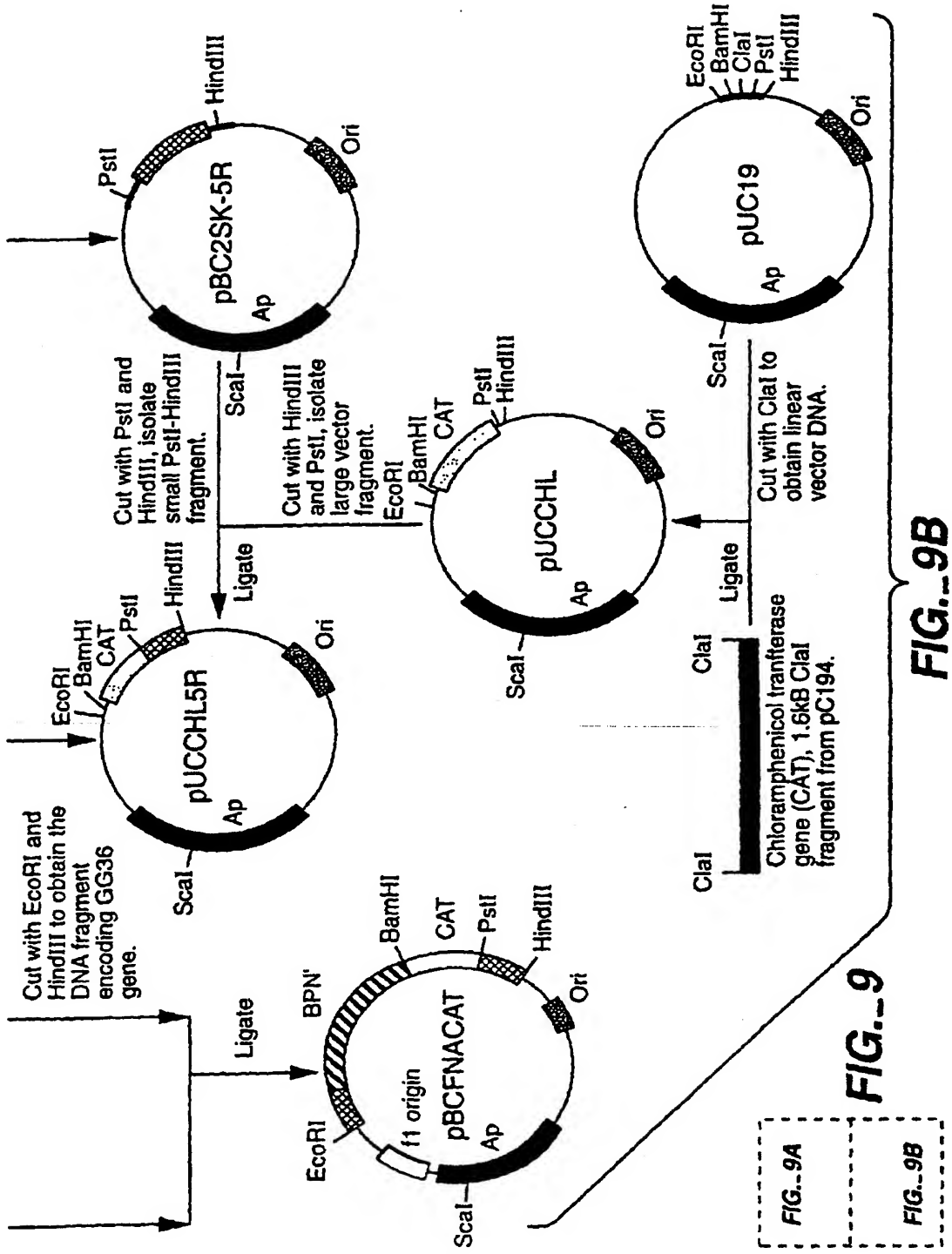

Cut with EcoRI and HindIII to obtain the DNA fragment encoding GG36 gene.
Ligate
GG36DAI
BamHI
CAT
PstI
HindIII
Ori
EcoRI
f1 origin
pBCDAICAT
Ap
ScaI
EcoRI
BamHI
CAT
PstI
HindIII
Ori
pUCCHL5R
Ap
ScaI
Cut with PstI and HindIII, isolate small PstI-HindIII fragment.
Cut with HindIII and PstI, isolate large vector fragment.
Ligate
PstI
HindIII
Ori
pBC2SK-5R
Ap
ScaI
EcoRI
BamHI
CAT
PstI
HindIII
Ori
pUCCHL
Ap
ScaI
Ligate
ClaI
ClaI
Chloramphenicol tranferase gene (CAT), 1.6kB ClaI fragment from pC194.
Cut with ClaI to obtain linear vector DNA.
EcoRI
BamHI
ClaI
PstI
HindIII
Ori
pUC19
Ap
ScaI
FIG._8A
FIG._8B
FIG._8
FIG._8B

FIG._9A


BS2KS+
f1 origin
EcoRI
HindIII
Ori
Ap
ScaI
Cut with EcoRI and HindIII to obtain the large vector fragment.
GGT274
DraI
GG36DAI
EcoRI
BamHI
CAT
Ap
pBR322ori
Cut with EcoRI and DraI to obtain small EcoRI-DraI fragment.
pBC2SK-
PstI
EcoRI
EcoRV
HindIII
Ori
Ap
ScaI
Cut with EcoRI and EcoRV to obtain linear large vector fragment.
Ligate
BPN'
EcoRI
BamHI
Cut with EcoRI and BamHI to obtain the large vector fragment.
Ligate
pUCCATFNA
BPN'
EcoRI
BamHI
CAT
PstI
HindIII
Ori
Ap
ScaI
pBC2SK-5
PstI
EcoRI
HindIII
Ori
Ap
ScaI
Cut with EcoRI, filled in with T4 DNA polymerase and dNTPs, and religate.


Cut with EcoRI and HindIII to obtain the DNA fragment encoding GG36 gene.
Ligate
BPN'
f1 origin
pBCFNACAT
Ap
Ori
EcoRI
BamHI
CAT
PstI
HindIII
ScaI
pUCCHL5R
pBC2SK-5R
pUCCHL
pUC19
Cut with PstI and HindIII, isolate small PstI-HindIII fragment.
Cut with HindIII and PstI, isolate large vector fragment.
Cut with ClaI to obtain linear vector DNA.
ClaI
Chloramphenicol tranferase gene (CAT), 1.6kB ClaI fragment from pC194.
FIG._9B
FIG._9
FIG._9A
FIG._9B

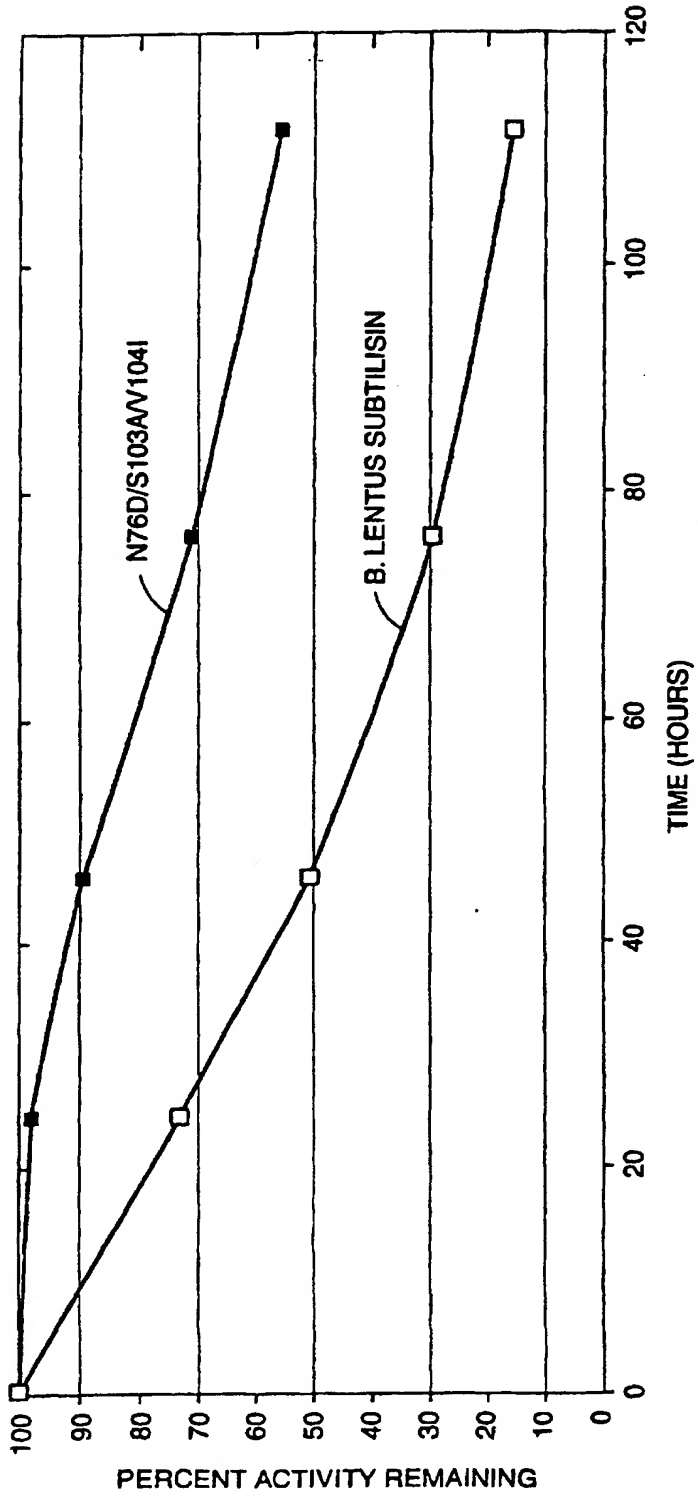

N76D/S103A/V104I
B. LENTUS SUBTILISIN
PERCENT ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
TIME (HOURS)
0
20
40
60
80
100
120

FIG._10